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Effect of Plasterboard Composition on *Stachybotrys chartarum* Growth and Biological Activity of Spores

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The effects of plasterboard composition on the growth and sporulation of *Stachybotrys chartarum* as well as on the inflammatory potential of the spores were studied. *S. chartarum* was grown on 13 modified plasterboards under saturated humidity conditions. The biomass was estimated by measuring the ergosterol content of the *S. chartarum* culture while the spore-induced cytotoxicity and production of nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and interleukin-6 in mouse macrophages was used to illustrate the bioactivity of spores. The ergosterol content of *S. chartarum* correlated with the number of spores collected from plasterboards. The growth and sporulation decreased compared to that of the reference board in those cases where (i) the liner was treated with biocide, (ii) starch was removed from the plasterboard, or (iii) desulfurization gypsum was used in the core. Spores collected from all the plasterboards were toxic to the macrophages. The biocide added to the core did not reduce the growth; in fact, the spores collected from that board evoked the highest cytotoxicity. The conventional additives used in the core had inhibitory effects on growth. Recycled plasterboards used in the core and the board lacking the starch triggered spore-induced TNF-α production in macrophages. In summary, this study shows that the growth of a strain of *S. chartarum* on plasterboard and the subsequent bioactivity of spores were affected by minor changes to the composition of the core or liners, but it could not be totally prevented without resorting to the use of biocides. However, incomplete prevention of microbial growth by biocides even increased the cytotoxic potential of the spores.

Plasterboard is a commonly used building material, since it has many favorable technical characteristics. It is composed of two paper liners sandwiching a core, containing gypsum, starch, and some additives. In the good practice of building design, construction, and maintenance, the structures should remain dry. However, in cases of moisture damage, the materials may be in contact with water for prolonged times. In such conditions, plasterboard may provide suitable nutritional conditions to permit the growth of microbes. The fungus *Stachybotrys* has been especially shown to occur often on moist and contaminated plasterboard (15). It has been suggested that the paper liner of plasterboard favors *Stachybotrys chartarum* growth because of its strong cellulolytic capacity (3, 10, 23). Our group has recently shown that *S. chartarum* growth on plasterboards is due not only to the liners but also to the core material, which plays an essential role (20). The growth of *S. chartarum* is often associated with adverse health effects in the occupants of buildings with mold problems (7, 14, 16). *S. chartarum* can produce several biologically potent mycotoxins such as trichothecenes (11, 21, 22, 23, 34), which are carried along with spores (24, 25, 27, 33). Our group has demonstrated that microorganisms, when grown on wetted plasterboards, can produce bioactive compounds capable of inducing a dose-dependent production of major inflammatory mediators such as nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) and capable of evoking cell death in mammalian cells (19).

In order to identify the critical components of plasterboard which can affect microbial growth with subsequent production of bioactive compounds, we grew *S. chartarum* on plasterboards of different compositions. The ergosterol content of *S. chartarum* culture on plasterboards was used to quantify the fungal biomass while spore-induced cytotoxicity and production of NO, TNF-α, and IL-6 in mouse macrophages were used to assess the bioactivity of spores.

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MATERIALS AND METHODS

Plasterboards. The plasterboards used in this study were prepared in cooperation with plasterboard manufacturers. The composition modifications of the 13 plasterboards are presented in Table 1. The plasterboard samples, 56 cm^2 in area, were sterilized (25 kGy gamma irradiation by Gammaster, Ede, The Netherlands) and wetted with 10 ml of sterile water prior to use.

Growth conditions. *S. chartarum* HT580 (National Public Health Institute, Kuopio, Finland) was isolated from a material sample of a mold-damaged building. The strain was identified morphologically at the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). *S. chartarum* was cultivated on 2% malt extract agar (Biokar Diagnostics, Beauvais, France) in the dark at 25°C for 7 days until it sporulated. The spores were collected with a sterile plastic rod and transferred to 10 ml of Hanks balanced salt solution (HBSS) (Gibco Laboratories, Paisley, United Kingdom). The number of spores in 2 ml of HBSS (10^6) counted by using a Bürker counting chamber (Tamro Medlab, Vantaa, Finland) was inoculated on the surfaces of the wetted plasterboard samples. Duplicate samples were placed in a tight, sterilized 4-liter glass vessel containing a thin layer of sterilized water on its bottom. To avoid direct contact with water, the pieces were placed on a rack standing above the water surface. Identically treated plasterboards without inoculations were used as controls. The vessels were kept at the room temperature of 18 to 22°C and aerated with filter-sterilized (0.2-μm pore size; Schleicher & Schuell GmbH, Dassel, Germany) air once per day for 10 min at a flow rate of 400 ml min^{-1}. After 4 weeks of *S. chartarum* growth, the microbiological and bioactivity analyses were performed.

Microbiological analyses. (i) Total spore numbers. The spores were collected from the inoculated area with a sterilized plastic rod and transferred to 10 ml of HBSS buffer. The spore concentration of *S. chartarum* suspensions was counted with a light microscope and Bürker counting chamber (Tamro Medlab). The

TABLE 1. Compositions of 13 different plasterboards

Modified component	Board no.	Composition modification by[a]:								
		NG	DSG	Recycled material	Starch	Additive 1[c]	Additive 2[d]	Additive 3[e]	Biocide[f]	Paper
Gypsum	1[b]	1	0	0	1	1	1	1	0	Normal
	2	0	1							
	3	0.9×		0.1×						
	4	0.5×		0.5×						
Starch	5				0					**No starch**[g]
	6				0					
	7				3×					
Additive	8					0				
	9						0			
	10							0		
	11				0	0	0	0		
Biocide	12								1	
	13									**Biocide**[h]

[a] Changes in composition in relation to that of the reference board are shown in boldface type. 1, presence of the component at the normal concentration; 0, component removed.
[b] Board 1 was used as the reference board. It contained a standard amount of NG, starch, and three different additives in the core, and it had a normal paper liner.
[c] Additive 1 was foam. The foam is mixed with gypsum slurry to give a lower density to the board.
[d] Additive 2 was an accelerator. The accelerator makes the setting time shorter, allowing for a higher production speed.
[e] Additive 3 was a water-reducing agent. The water-reducing agent allowed us to get the required rehological properties of the slurry with less water.
[f] The biocide was 1% Parmetol DF 17 (Schülke & Mayr UK Ltd.).
[g] Normal paper but no starch.
[h] Normal paper treated with 1% Parmetol DF 17.

spore concentrations were expressed as numbers of spores per surface area of the plasterboard sample.

(ii) Ergosterol analysis. The determination of the ergosterol content of *S. chartarum* grown on different plasterboards was based on previously published methods (4, 28). Briefly, ergosterol standards (Sigma, St. Louis, Mo.) and inoculated plasterboard samples were heated with 10% KOH in methanol. A 7-dehydrocholesterol (Sigma) was used as an internal standard. The samples were purified on a silica gel column. Ergosterol was trimethylsilyl (TMS) derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Fluka Chemie, Buchs, Switzerland) and analyzed with a Hewlett-Packard Co. (Palo Alto, Calif.) model G1800A gas chromatograph equipped with a mass-selective detector and an HP7673 automatic sampler. The gas chromatography conditions were as follows: HP-5 capillary column (30 m by 0.2 mm by 0.11 μm) coated with cross-linked 5% Ph Me Silicone; carrier gas, helium (flow rate, 1.0 ml min^{-1}); injection, splitless; injector temperature, 290°C; detector temperature, 300°C. The oven temperature was programmed to hold 60°C for 1 min and to then increase by 25°C min^{-1} up to 290°C. The mass selective detector was operated in the selected ion-monitoring mode. The *m*/*z* 337 and 363 ions for the ergosterol TMS derivative and the *m*/*z* 325, 351, and 456 ions for the 7-dehydrocholesterol TMS derivative were used in the quantifications. The ergosterol concentrations were expressed as micrograms of ergosterol per surface area of the plasterboard sample. The lowest detectable amount of ergosterol was about 10 pg/cm.

Bioactivity analyses. (i) Treatment of RAW264.7 cells. The mouse macrophage cell line RAW264.7 was originally purchased from the American Type Tissue Collection (Rockville, Md.). The cells were grown at 37°C in 5% CO_2 in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah), 1% L-glutamine, and 1% penicillin-streptomycin antibiotic mixture (Gibco Laboratories). The cells were then exposed for 24 h to the spores of *S. chartarum* collected from plasterboard samples at the doses of 10^4, 10^5, and 10^6 spores/10^6 cells.

(ii) Nitric oxide analysis. After 24 h of incubation, NO was assayed in the cell culture medium by measuring the stable NO oxidation product nitrite (NO_2^-) by using the method based on the Griess reaction (12). A 50-μl aliquot of cell culture medium and standards were incubated in duplicate with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid) on a 96-well microtiter plate (Maxisorb; Nunc, Naperville, Ill.). The chromophore absorbance at 540 nm was determined with a microplate reader (iEMS Reader MF; Labsystems, Espoo, Finland). The nitrite concentration was assessed by using sodium nitrite as a standard. The lowest detectable nitrite concentration was about 500 pg/ml.

(iii) Cytotoxicity. The cytotoxicity of the spores in the cell culture was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,3-dimethyltetrazolium bromide (MTT) test (Sigma), where exogenously administered MTT solution is converted to the colored formazan via intact mitochondria of the cells, which can be photometrically determined (18). Briefly, after 24 h of incubation, a cell suspension and MTT solution were added in duplicate to each well in the 96-well plate. After 2 h of incubation, sodium dodecyl sulfate buffer was added. After an overnight incubation, the absorbance at 570 nm was measured with a microplate reader (iEMS Reader MF; Labsystems). The viability of the cells was calculated in relation to the viability of the control cells.

(iv) Cytokine analysis. After 24 h of incubation, TNF-α and IL-6 concentrations were measured from cell culture medium by using enzyme-linked immunosorbent assay kits (R & D Systems, Minneapolis, Minn.) according to the manufacturer's instructions. The lowest detectable cytokine concentrations were about 5 pg/ml.

Composition analyses. Elementary compositions of plasterboard core materials were analyzed by inductively coupled plasma atomic emission spectrometry with a Thermo Jarrell Ash Iris/Dual View (Thermo Elemental, Franklin, Mass.) after hot (+90°C) aqua regia digestion (26). For analyses of nutrient contents and pHs of plasterboard cores, 10 g of core material was eluted with 75 ml of ion-exchange-purified water (Millipore Corp., Bedford, Mass.) three times. From this solution, the content of assimilable organic carbon was determined according to the methods of van der Kooij et al. (35) and the American Public Health Association (1). The nitrate concentration was determined spectrophotometrically with flow injection analysis according to the method of the Finnish Standards Association and European Standard and International Organization for Standardization 13395 (SFS-EN ISO 13395). Ammonium and phosphate concentrations were determined spectrophotometrically according to Lachat QuikChem methods 10-107-06-1-F and 10-115-01-1-Q (Lachat Instruments Divison, Zellweger Analytics Inc., Milwaukee, Wis.). The pH of the solution was measured with an Orion Research (Cambridge, Mass.) 611 pH millivolt meter.

Statistical analyses. Statistical analyses of data were performed by using SPSS for Windows, version 10.1 (SPSS, Inc., Chicago, Ill.). Because the data were nonnormally distributed, the correlations between ergosterol content and the amount of spores were studied by using Spearman correlation analysis. One-way analysis of variance and Dunnett's test were used for comparing ergosterol and spore contents as well as the biological activity of spores of the sample boards to that of spores of the reference board. The accepted level of statistical significance was a P value of ≤ 0.05.

RESULTS

Microbial growth. The biomass production of *S. chartarum* estimated as ergosterol content per area as well as the spore number per area, which were obtained from the sampled area of 56 cm^2, varied between plasterboards with different compo-

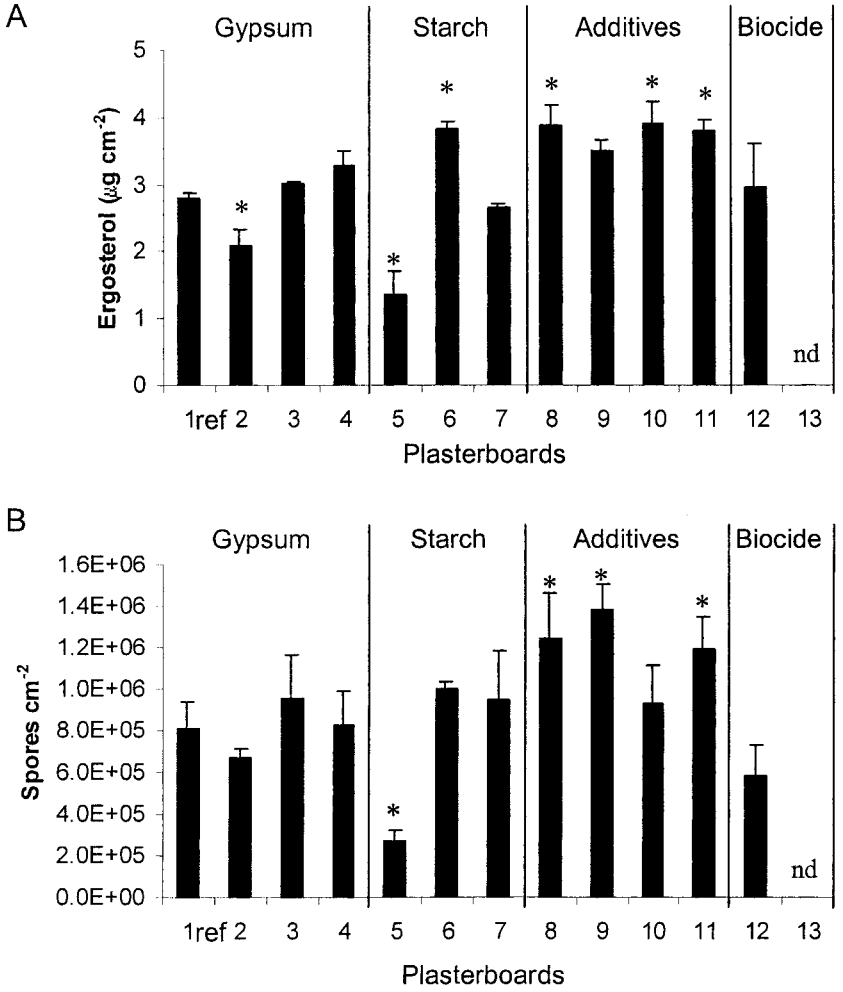

FIG. 1. Ergosterol content (A) and number of spores (B) of *S. chartarum* after 4 weeks of growth on different plasterboards. Gypsum, starch, additive, and biocide contents were changed according to the listings in Table 1. Plasterboard 1 was used as a reference board. Each column represents the mean ± standard deviation of duplicate samples. Asterisks indicate significant differences from reference board 1 ($P \leq 0.05$). nd, not detected. The lowest detectable amount of ergosterol was about 10 pg/cm^2.

sitions. There was a statistically significant correlation between the ergosterol content and the amount of spores ($r > 0.76$, $P \leq 0.03$) (Fig. 1). The biomass production was significantly decreased on boards 2, 5, and 13 while it was increased on boards 6, 8, 10, and 11 when compared with reference board 1 (Fig. 1A). The amount of spores was decreased on boards 5 and 13, whereas it was increased on boards 8, 9, and 11 (Fig. 1B). The plasterboard with a liner treated with 1% Parmetol DF 17 (board 13) inhibited *S. chartarum* growth and sporulation completely. On the contrary, the addition of the biocide to the core (board 12) did not prevent the growth, and it only slightly decreased the spore number. Plasterboard without any starch (board 5) did not support abundant growth or sporulation (Fig. 1). Instead, if the starch was removed only from the core (board 6), the amount of biomass and number of spores increased. The addition of starch to the core (board 7) did not affect the sporulation or growth when compared to the reference board. When desulfurization gypsum (DSG) was used in the core (board 2), the amount of biomass and number of spores were reduced, whereas the use of recycled plasterboards in cores (boards 3 and 4) slightly increased the biomass production. The levels of nutrients and trace metals, excluding arsenic and lead, in boards with DSG were considerably lower when compared with those of the reference board or boards with recycled plasterboards (Fig. 2). Removal of any or all of the additives (boards 8 to 11) increased the sporulation and biomass production (Fig. 1). The pH values of cores were fairly constant, varying in the range of 7.10 to 7.82. Neither ergosterol nor spores were detected in control boards.

Biological activity. *S. chartarum* spores collected from tested plasterboards induced bioactive responses in mouse macrophages, assessed as cytotoxicity and production of IL-6 and TNF-α in cell culture medium, in a dose-dependent manner (Fig. 3). The spores collected from all tested plasterboards, apart from board 13, evoked severe cytotoxicity up to 90.9% in mouse macrophages at the highest tested dose (10^6 spores/10^6 cells) in comparison to untreated control cells (Fig. 3A). *S. chartarum* inoculated on board 13, the liner of which had been treated with biocide, did not grow or sporulate, and therefore, macrophage exposure could not be tested in this case. Instead,

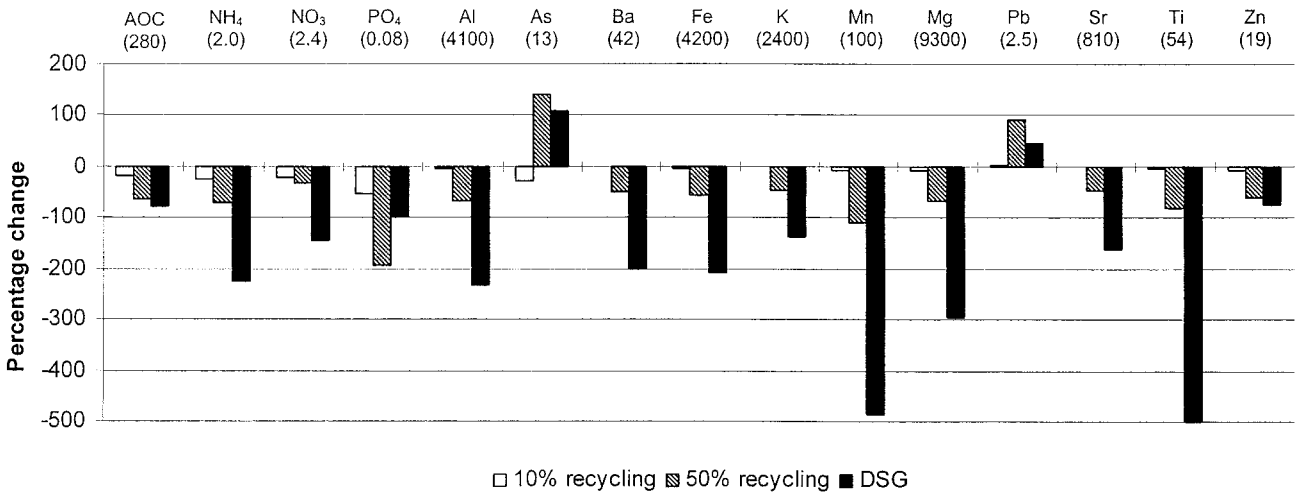

FIG. 2. Percent change of nutrient and trace metal contents of recycled (10 and 50%) plasterboards and DSG used in core materials in comparison to reference plasterboard 1 (Table 1). Nutrient and trace metal contents of reference board 1 are presented in parentheses (in milligrams per kilogram).

the macrophage exposure testing was carried out with the spore collection medium, but it did not induce any cytotoxic or inflammatory responses. Plasterboard 12, with biocide in the core, decreased sporulation, but the spores that were produced were extremely cytotoxic. The spores collected from boards 2 and 4, with DSG and with 50% recycled plasterboards in the core, respectively, evoked significantly lower cytotoxicities than did the spores from reference board 1. However, they still killed more than 50% of the cells.

Spores collected from boards with recycled plasterboard in the cores (boards 3 and 4) and from boards without any starch (board 5) induced increased TNF-α production in mouse macrophages, whereas spores from board 3 and from a board without additive 2 (board 9) increased IL-6 production compared with reference board 1 (Fig. 3, B and C). *S. chartarum* spores did not induce any NO production at any tested dose collected from any of the tested boards (data not shown). Surface samples collected from control plasterboards that did not contain any spores induced cytotoxicity and inflammatory responses at about the same level as the lowest spore dose, 10^4 spores/10^6 cells (data not shown).

DISCUSSION

The present study shows that the growth of *S. chartarum* on plasterboard and the subsequent bioactivity of spores can be affected by changing the composition of the board core or liner. However, growth still occurs if only one of the main plasterboard components is altered at a time. *S. chartarum* is a cellulose-decomposing fungus that grows well on cellulose-rich substrates, such as straw and hay, which also support the production of several mycotoxins (13). Cellulose-rich paper liners of plasterboards have been suggested to be the main nutrient source for growth of *Stachybotrys* on this building material (3, 10, 23). This study demonstrates that the composition of paper liners has an impact on *S. chartarum* growth. No reduction of growth occurred due to the removal of starch from the core alone (board 6); growth reduction only occurred when the starch was removed both from the core and the liner (board 5). This indicates that, in addition to cellulose, the starch in the liner is also an important nutrient source for *S. chartarum* growth. Parmetol DF 17, used as a biocide, prevented *S. chartarum* growth and sporulation only when it was added to the liner (board 13). When biocide was added only to the core (board 12), growth still occurred to some extent. However, the spores collected from that board were highly toxic. In addition, after the poor growth on the starch-free plasterboard, the spores induced intense TNF-α production in macrophages. This suggests that under these conditions *S. chartarum* is able to produce bioactive secondary metabolites such as mycotoxins in response to growth inhibition or nutrient starvation. There are several different preservatives and biocides which have been used to inhibit fungal growth, including that of *S. chartarum*, on gypsum-containing building materials (29, 32). However, there are some aspects that should be taken into account with respect to the use of growth inhibitors. When using biocides that affect multiple targets at low, growth-inhibitory concentrations, the susceptibility of each target is variable and dependent on the concentration of the biocide (6). Therefore, it is necessary to assess the extent to which exposure of microbial populations to biocides results in reduced biocide susceptibility. Furthermore, it has been suggested that widespread use of biocides in consumer products may have a role in the development of antibiotic resistance in clinical practice (17, 30).

There is convincing evidence that cellulose-rich paper liners support *S. chartarum* growth. However, in a comparison of different types of moisture-damaged building materials, Hyvärinen et al. (15) found that 30% of plasterboard samples and only 10% of paper samples were contaminated with species of *Stachybotrys*. This is in line with previous conclusions that the core material of plasterboard also has an essential role in *S. chartarum* growth (20). The nutritional composition of the core material is dependent on the source of gypsum. This was demonstrated when the amounts of nutrients in the cores decreased along with the increased amount of recycled plaster-

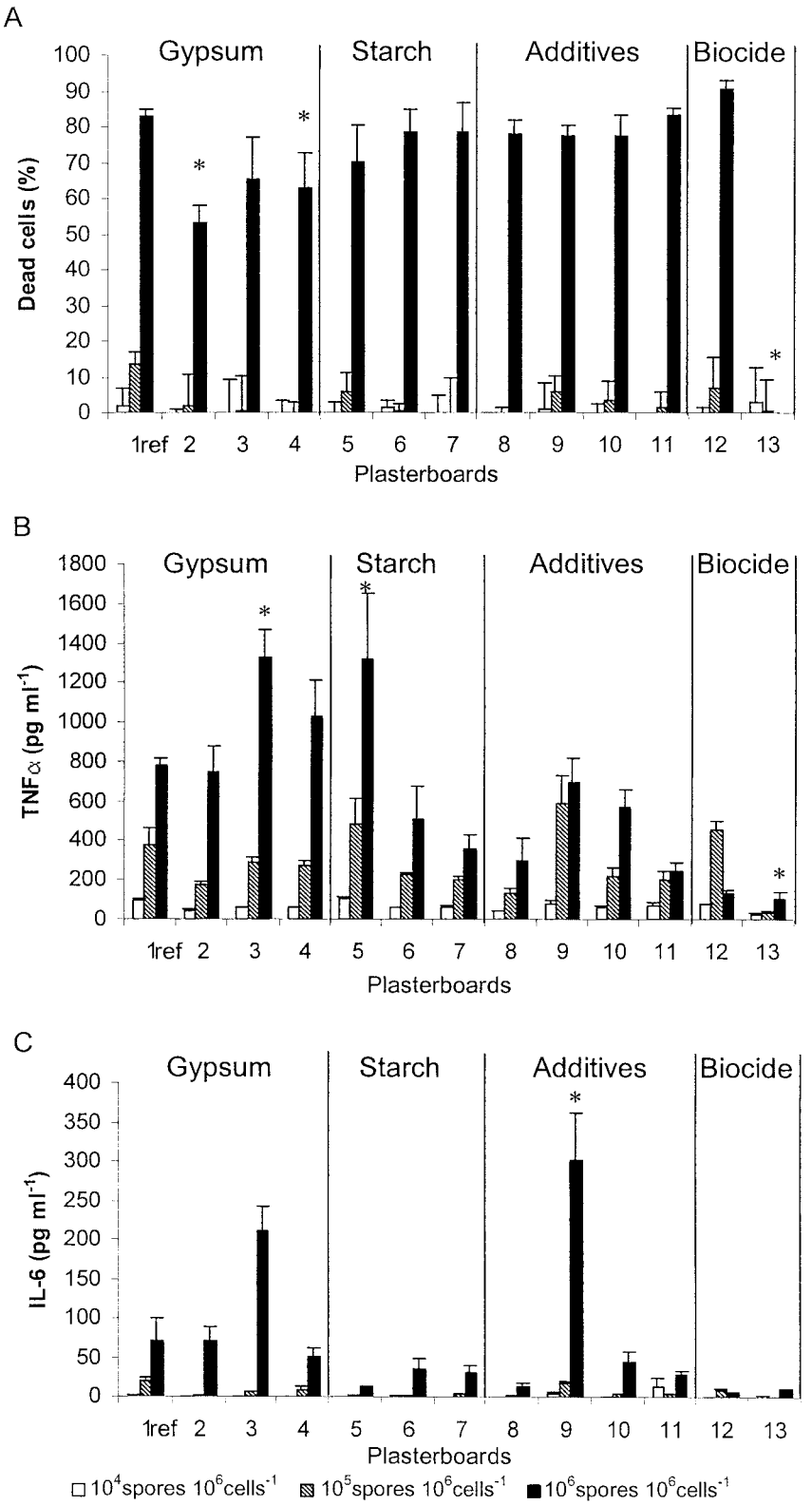

FIG. 3. Cytotoxicity (A) and production of TNF-α (B) and IL-6 (C) in RAW264.7 macrophages induced by three different doses of *S. chartarum* spores collected from different plasterboards. Gypsum, starch, additive, and biocide contents were changed according to listings in Table 1. Plasterboard 1 was used as a reference board. Each column represents the mean ± standard error of the mean of three independent experiments performed in duplicate. Asterisks indicate significant differences from reference board 1 ($P \leq 0.05$). The lowest detectable cytokine concentrations were about 5 pg/ml.

board used. Also, the nutrient content of DSG was markedly lowered in comparison with that of natural gypsum (NG). In order to grow, fungi require sources of carbon and nitrogen, a supply of energy, and certain essential nutrients such as potassium and phosphorus. The quantities of nutrients such as assimilable organic carbon, NO_3, and PO_4 eluted from the reference board core were relatively low (280, 2.4, and 0.08 mg/kg, respectively) when compared, for example, with standard Czapek agar medium (31), which contains about 30 g of sucrose/liter, 2.2 g of NO_3/liter, and 0.5 g of PO_4/liter. The contents of these nutrients were still considerably lower in the DSG. On the basis of these results, it seems that the weak growth of *S. chartarum* on the DSG board (board 2) may be at least partially due to its low nutrient content. Interestingly, spores collected from the DSG board also evoked the lowest cytotoxicity.

Similarly, as in case of the above nutrients, the amounts of certain inorganic ions such as Al, Fe, K, Mg, and Mn were systematically reduced in DSG board in comparison with the reference board. In contrast to the nutrients, the quantities of these ions were so high that they cannot be considered to limit the growth. In line with these findings, Andersen and Nissen (2) have shown that cereal- and vegetable-based growth media, rather than mineral-based media, support *S. chartarum* growth and sporulation. On the other hand, the elementary analysis of boards, including acidic aqua regia digestion, might overestimate the biologically available metal concentration since it also liberates cations that are probably precipitated at the alkaline pH present in the cores (8). While the contents of most of the heavy metals were decreased in boards with DSG or recycled plasterboard, the amount of arsenic and lead actually increased. These metals are known to be strongly inhibitory for microorganisms at relatively low concentrations (9). However, the concentrations of these heavy metals in the studied plasterboards are much lower than their concentrations in contaminated soils where they have been shown to reduce microbial growth (5). Moreover, the highest amounts of these metals were detected from the board containing 50% recycled plasterboard (board 4), which supported growth better than the board with DSG. Thus, the growth differences on plasterboards are likely not to be accounted for by heavy metal toxicity.

These results demonstrate that growth of a strain of *S. chartarum* on plasterboard under saturated humidity conditions was affected by modifications made to the plasterboard composition. The growth was reduced on boards where (i) the liner was treated with 1% Parmetol DF 17, (ii) starch was removed from the plasterboard, or (iii) DSG was used in the core. The plasterboard composition also has effects on the biological activity of the spores; however, the effects were not consistent with growth. When a manufacturer is considering which raw materials to include in its plasterboard, it should take into account that the nutritional composition of gypsum varies and this has an impact on microbial growth. If biocides are used, it is important to assess the concentration as well as the persistence of the chemical used in order to achieve the appropriate antimicrobial function. This study concerned the effects of plasterboard components on the growth of *S. chartarum*, which is only one of the microbial species that affects the quality of indoor air. There are several other important microbes that it would also be necessary to study. Finally, the effects of different types of paper liners on microbial growth and on spore-induced bioactivity need to be characterized in more detail to get an overall perspective on the microbiological characteristics of plasterboard.

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